

A New Type of the Manifestation of Colony Specificity in the Compound Ascidian, *Botrylloides violaceus* Oka¹

EUICHI HIROSE, YASUNORI SAITO, AND HIROSHI WATANABE

Shimoda Marine Research Center, University of Tsukuba, Shimoda 5-10-1, Shizuoka 415, Japan

Abstract. A new type of colony specificity (= allogeneic recognition) is shown for *Botrylloides violaceus*. All botryllid ascidians previously studied for colony specificity show allo-recognition reactions, manifested as fusion or nonfusion (rejection), both at the colonial margin (= growing edge) and at the cut surface. By contrast allo-recognition in *Botrylloides violaceus* is absent at the cut surface, but present at the growing edge. Juxtaposition of cut surfaces resulted in fusion of the colonies regardless of origin, while juxtaposition of natural growing edges resulted in fusion or rejection, according to the genetic combination of colonies. Similar results occurred among the sibling colonies derived from the same mother colony, in which pairs tunic necrosis was observed in areas where the two colonies partially fused. These features of allogeneic rejection in *B. violaceus* were very similar to those of "nonfusion" in *Botrylloides simodensis*. In inter-specific combinations between *B. violaceus* and *B. simodensis*, a remarkable necrotic reaction was observed in the zone of contact when two colonies touched at their cut surfaces. When brought into contact at their growing edges, they resulted in "nonfusion" without a particular reaction.

Introduction

Colony specificity represented by allograft rejection has been demonstrated for many colonial forms of animals, from sponges to ascidians. Colony specificity in some compound ascidians is manifested by fusibility between colonies; two colonies either form a single mass (fusion) or do not fuse (rejection), when they come into contact. Some ascidian species do not show colony speci-

ficity, while others exhibit it (Koyama and Watanabe, 1982). In those that do, isogenic colonies are always fusible at their natural growing edges, and allogeneic colonies are either fusible or not fusible.

Colony specificity in compound ascidians has been studied primarily in species of the family Botryllidae (botryllid ascidians). These species form sheet-like colonies in which zooids are buried in a gelatinous tunic. Zooids are arranged in rosettes or ladder-like systems with common cloacal apertures, and are connected to one another by a ramifying network of blood vessels which terminate in sausage-shaped *ampullae* at the periphery of the colony. All botryllid ascidians that have been studied so far exhibit colony specificity. In some of them, genetic control of their fusibility has been demonstrated (Oka and Watanabe, 1957, 1960, 1967; Sabbadin, 1962, 1982; Scofield *et al.*, 1982).

The morphology and cell biology of fusion and nonfusion (rejection) in botryllids have been studied in detail in four species: *Botryllus scalaris* (Saito and Watanabe, 1982), *B. primigenus* (Oka and Watanabe, 1967; Tanaka and Watanabe, 1973; Tanaka, 1973; Katow and Watanabe, 1980; Taneda and Watanabe, 1982), *B. schlosseri* (Milanesi *et al.*, 1978; Scofield and Nagashima, 1983), and *Botrylloides simodensis* (Mukai and Watanabe, 1974; Saito, 1976; *cf.* Saito *et al.*, 1981). The course of fusion is essentially the same in all these species. By contrast, the rejection reaction is initiated at distinctly different stages of fusion in different species. These facts imply that comparative studies of the processes of fusion and nonfusion in various species might be useful for analyzing the mechanism of allo-recognition, as well as for considerations of the evolution of colony specificity in botryllid ascidians. Here we have investigated the processes of fusion and nonfusion in the Japanese species *Botrylloides violaceus*, and found that this species shows

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Table 1

Fusion experiments in Botrylloides violaceus at the cut surface

Combination	No. combination	Fusion	Nonfusion
Between colonies of the Shimoda population	112	112	0
Between colonies of the Asamushi population	27	27	0
Between the two populations	33	33	0

a new and instructive type of colony specificity previously undescribed for compound ascidians.

Materials and Methods

Colonies of *B. violaceus* were collected in the Shimoda Floating Aquarium (Shizuoka Prefecture) and in the vicinity of the Asamushi Marine Biological Station (Aomori Prefecture). The two collecting sites are about 1100 km apart in linear distance. *B. simodensis* colonies were also collected in the vicinity of the Shimoda Marine Research Center (Shizuoka Prefecture). To facilitate handling of the colonies, they were fixed on glass plates and reared in the culture boxes floated in the bay near the Shimoda Marine Research Center.

The fusibility of colonies was tested by fusion experiments. Procedures for fusion experiments were as follows: a piece was cut out of each of the two colonies between which fusibility was to be tested. The two similarly sized pieces (about 15 zooids) were placed in juxtaposition on a glass slide, and they were brought into contact with each other either at the cut surfaces or at the growing edges. The colonies on the glass slide were then kept in a moisture chamber for 30 minutes, so that the colonies might attach to the glass slides prior to their placement in running seawater. The experimental animals were observed under a binocular stereomicroscope each day. The paired colonies showed clear fusion or nonfusion reactions. Fusion here means the establishment of a common vascular system between the two colonies, and nonfusion the absence of it. In the latter case, several trials with the same combination of colonies were carried out to avoid the possibility of accidental failure of fusion.

Oozoids of *B. violaceus* were obtained from some colonies from the Shimoda Floating Aquarium population. In the fusion experiments of sibling colonies, a pair of oozoids derived from the same mother colony were placed on a glass slide so that they came into contact by their growing edges. Further experimental methods were the same as those just mentioned.

For histological studies, specimens in the process of fusion or nonfusion were fixed in a solution containing 2.5% glutaraldehyde and 0.45 M sucrose buffered with

0.1 M cacodylate at pH 7.4. The fixed specimens were then dehydrated through a butanol series, embedded in paraplax, sectioned at 5 μ m and stained with Congo red, Delafield's hematoxylin, and eosin-orange G.

Results

Fusion experiments at the cut surface

When colonies of *Botrylloides violaceus* were apposed by their cut surfaces, we were surprised to note that all colonies fused (Table I). This was true both in intrapopulation and in interpopulational combinations. However, separation of such fused colonies between which the common vascular system had been established for a few days or more was occasionally observed. This suggests the presence of a kind of long term (or induced) allo-recognition. It should be noted, however, that in these experiments it was difficult to determine whether separation was caused by allo-recognition or by unsuitable conditions for long-term observation. By contrast, a rapid, necrotic xeno-graft rejection was observed between similarly juxtaposed pieces of *B. violaceus* and *B. simodensis* colonies. Blood cells from the blood vessels clustered in the tunic at the boundary between the colonies. The clusters of cells were observed as a clear *black line* along the boundary (Fig. 1). The area with the cell infiltration eventually degenerated and was apparently cleared from the area.

Fusion experiments at the growing edge

By contrast to the results with cut colonies, apposition of colonies by their naturally growing edges resulted either in fusion or nonfusion, depending on the particular combination of colonies employed. In the case of fusion,

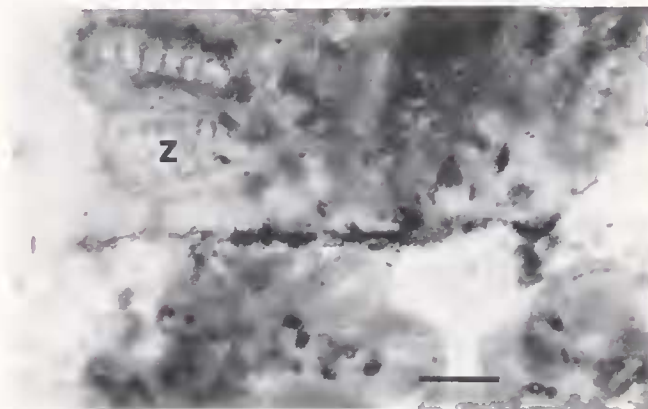


Figure 1. Xeno-rejection at the cut surface. Upper colony is *Botrylloides violaceus*, and lower colony is *B. simodensis*. Necrosis is observed along the boundary between the two colonies. z, zooid. Scale bar = 1 mm.

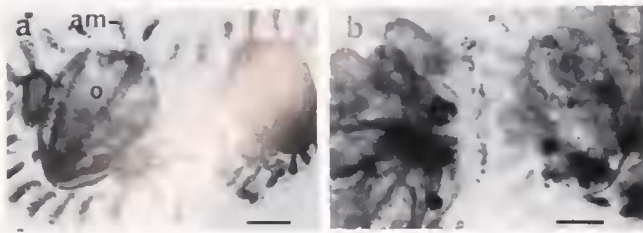


Figure 2. Fusion and nonfusion at the growing edge between sibling colonies of *Botrylloides violaceus*. a: Fusion, four days after settlement of the oozoids. Common vascular system has been established. b: Nonfusion, a week after settlement. am, ampullae; o, oozoid. Scale bars = 0.5 mm.

tip to side contacts occurred between ampullae of the two colonies, and then fusion took place at those sites to produce a common vascular system. In the case of nonfusion, ampullae of both colonies pushed against each other, but never penetrated the tunic of the facing colony. Although signs of rejection were not clearly visible in the contact area under the binocular stereomicroscope, the necrotic rejections were evident in subsequent histological sections of the rejection zone (below).

Similar experiments between sibling oozoids gave similar results (Fig. 2). Out of the 32 combinations studied, 14 resulted in fusion and 10 resulted in nonfusion. The results of the remaining eight combinations could not be assessed because of the degeneration or mechanical separation of the colonies.

All xenogeneic combinations between *B. violaceus* and *B. simodensis* resulted in nonfusion reactions similar to those in incompatible intraspecies pairs.

Histological observations

A frontal section of normal ampullae at the periphery of a colony is shown in Figure 3. The ampullae are buried in the tunic. Many blood cells are seen in the ampullar lumen, and are not usually seen in the tunic. Tunic (or "test") cells are dispersed throughout the tunic, and can be distinguished from blood cells by their morphology and staining characteristics. A cuticular layer is differentiated at the external surface of the tunic.

The histological features of fusion and nonfusion at the growing edge of *B. violaceus* were very similar to those of *B. simodensis* as reported by Saito (1976). In the case of fusion the boundary between the two colonies

disappeared and was filled with continuous test matrix containing normally distributed tunic cells (Fig. 4a-c). Fragments of cuticle were sometimes observed in the original boundary zone (Fig. 4b). In the case of nonfusion, the tunic layers sometimes fused, but only in small areas along the boundary. In the fused areas, tunic cells were considerably more abundant than usual (Fig. 4d). In addition, blood cells (particularly morula cells) infiltrated the tunic from the blood vessels in the contact areas (Fig. 4e). These cells were found clustered and disintegrated in the rejection zone. The disintegrated cells subsequently were released from the tunic in the form of massive aggregations (Fig. 4f).

Discussion

In *Botrylloides violaceus*, short term allo-recognition appears to be absent at the cut surface and present at the growing edge. This type of colony specificity has not been described previously. In compound ascidians previously studied, mechanisms of allo-recognition have been studied in *Didemnum moseleyi* (Mukai and Watanabe, 1974), *Perophora japonica* (Koyama and Watanabe,

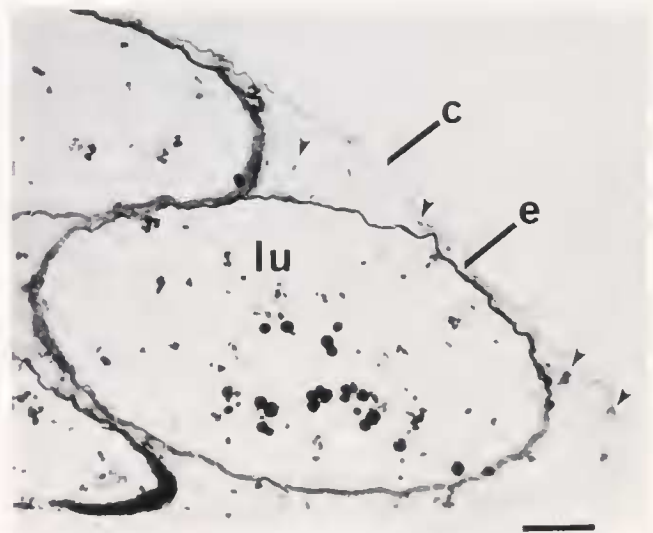
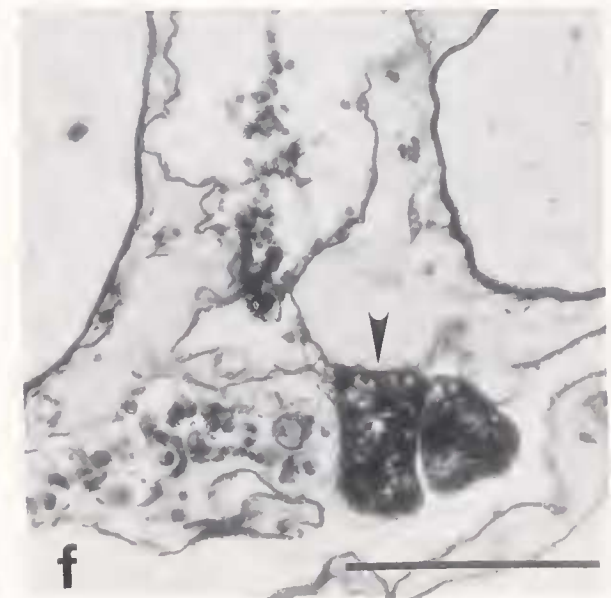
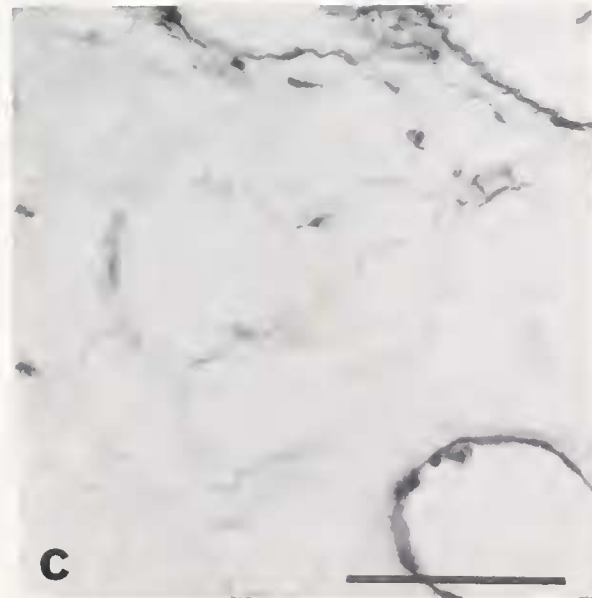
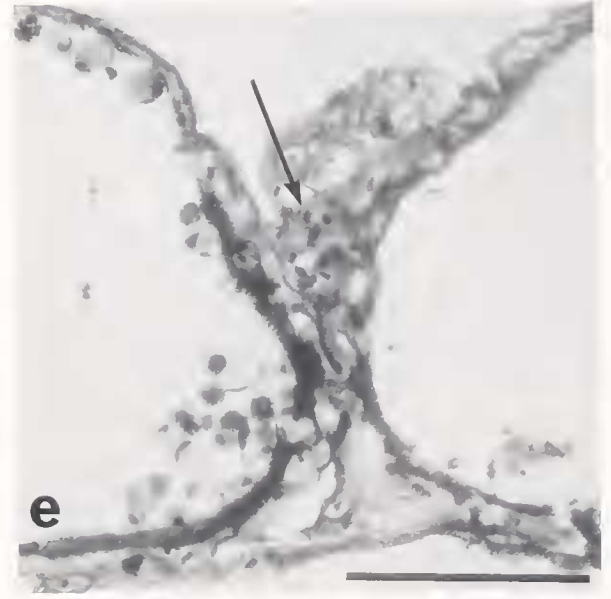
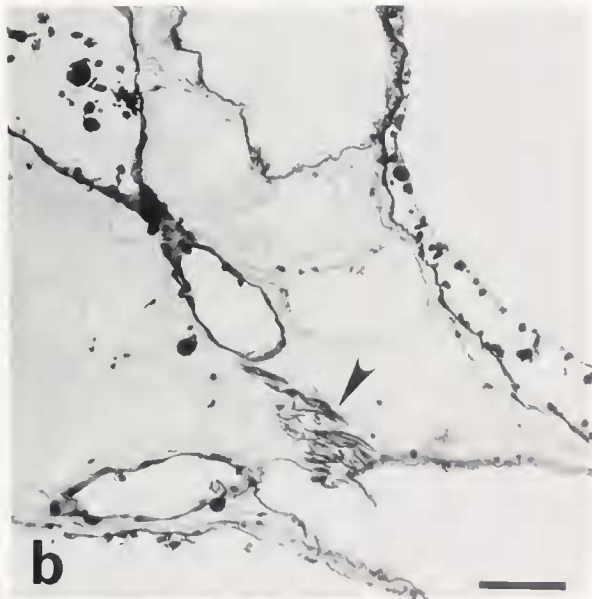
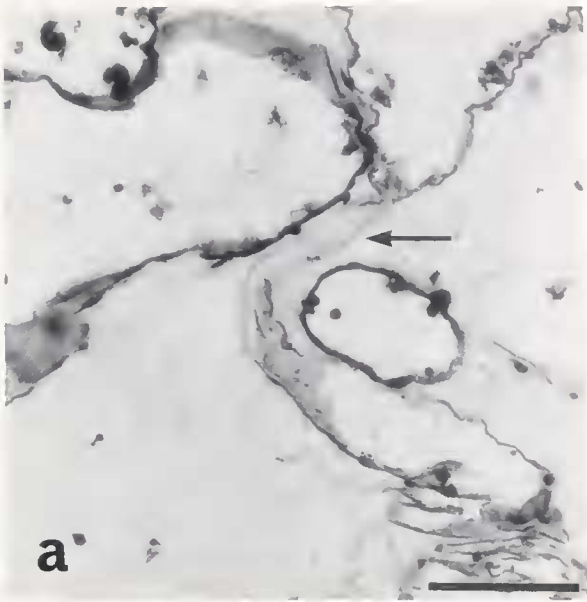


Figure 3. Frontal section through the periphery of a colony of *Botrylloides violaceus*. The substratum is to the bottom and the colony is growing to the right. Some of the tunic cells are pointed by arrow heads. c, cuticle; e, epithelium of ampulla; lu, lumen of ampulla with blood cells. Scale bar = 50 μ m.

Figure 4. Histological aspects of fusion and nonfusion at the growing edge between sibling colonies of *Botrylloides violaceus*. a-c: Fusion. (a) Contact of colonies. Dissolution of cuticular layers occurs at the contact area (arrow). (b) Fusion of colonies. Fragments of cuticle are still observed (arrow head). (c) Completely fused tunic. d-f: Nonfusion. (d) Partially fused tunic in which there are many tunic cells. (e) Blood cells infiltrate into the tunic and form a mass of cells (arrow heads). (f) The mass of destructed cells is removed to the outside of the tunic (arrow head). Scale bars = 50 μ m.



1981), *P. sagamiensis* (Koyama and Watanabe, 1982), *Symplegma reptans* (Mukai and Watanabe, 1974), *Botryllus scalaris* (Saito and Watanabe, 1982), *B. primigenus* (Oka and Watanabe, 1957), *B. schlosseri* (Bancroft, 1903; S. Taneda, 1962; Scofield and Nagashima, 1983), and *Botrylloides simodensis* (Mukai and Watanabe, 1974). Although allo-recognition at the cut surface is uncertain in the two species of *Perophora* and *B. schlosseri*, all the other ascidians listed clearly have the capacity for allo-recognition, both at the cut surface and at the growing edge (reviewed by Watanabe and Taneda, 1982; Taneda *et al.*, 1985). On the other hand, in the species we and others found lacking colony specificity, e.g., *Polycitor proliferus* (Oka and Usui, 1944; cf. Tokioka, 1953) and *Perophora multiclathrata* (Mukai and Watanabe, 1974; cf. Nishikawa, 1984), two colonies never fuse naturally at their growing edges, but invariably fuse at their cut surfaces, regardless of their origin. In *B. violaceus*, a colony invariably fuses with any other member of the same population or of another population at the cut surface. At the growing edge, however, colonies show fusion or nonfusion depending on the combination of colonies. From this we conclude that *B. violaceus* has a new type of colony specificity.

The nonfusion reaction observed at the growing edges of *B. violaceus* is similar to that of *B. simodensis*, which sometimes has been called "indifference." This term means nonfusion without particular reaction, such as the nonfusion of the ascidians lacking colony specificity, e.g., *Perophora multiclathrata* (Mukai and Watanabe, 1974). In the nonfusion of both *B. simodensis* and *B. violaceus*, the necrotic reaction between two colonies is barely observable under a binocular stereomicroscope, but is clearly observed histologically. Consequently, the nonfusion of these species should be placed in a category other than indifference. We propose, therefore, to use the term "sub-cuticular rejection" to describe nonfusion reactions in these two species. In sub-cuticular rejection, the necrotic reaction with blood cell infiltration is limited to the tunic along the boundary between colonies, and never occurs in the ampullae and blood vessels.

In sub-cuticular rejection, allo-recognition appears to occur in the sub-cuticular region of the colonial margin because the reaction is limited to that area. In fact, *B. violaceus* may conduct allo-recognition *only* in the sub-cuticular region, since allo-recognition is absent at the cut surface in this species. The sub-cuticular region of tunic consists of tunicin fibers and tunic cells. Therefore, the tunic cells might play an important role for allo-recognition.

All botryllid ascidians studied with regard to colony specificity are capable of natural allo-recognition at the growing edge. The fusion reaction is essentially the same in all species studied, but the nonfusion reaction is initi-

Table II

The stage at which the nonfusion reaction is initiated at the growing edge

Species	Stage	Reference
<i>B. scalaris</i>	ampullar fusion	Saito and Watanabe, 1982
<i>B. primigenus</i>	ampullar penetration	Taneda and Watanabe, 1982
<i>B. simodensis</i>	partial fusion of tunic	Saito, 1976
<i>B. violaceus</i>	partial fusion of tunic	

ated at different stages of the fusion process according to species (Table II). Taneda and his colleagues (1985) suggested that these differences depend upon the site in which allo-recognition initially occurs, and that the site has shifted to the surface of the colony during evolution. The nonfusion reaction initiated after ampullar fusion (in *B. scalaris*) seems to be a fundamental (or "primitive") type of allo-recognition in botryllids, and sub-cuticular rejection (in *B. violaceus* and *B. simodensis*) may be more advanced. Several species of botryllids, including *B. scalaris*, *B. primigenus*, *B. simodensis*, and *B. violaceus*, have also been studied in detail from the view point of life history (Saito and Watanabe, 1985), and their likely phylogenetic relationships have been outlined using rejection type as a reference point (Taneda *et al.*, 1985). From the presence of subcuticular rejection in this species, we deduce that *B. violaceus* may be an "advanced" species.

In *B. violaceus*, colony specificity is absent at the cut surface, although it is present at the growing edge. In this species, it is possible that the cells which have the capacity for allo-recognition distribute restrictedly in the sub-cuticular region. Therefore, in the case of cut surface contact, the cells having the capacity may not be exposed to allogeneic tissues, since the subcuticular region has been cut off. Then, the fusion would be allowed between allogeneic colonies. In the other botryllids, allo-recognition at the cut surface seems to occur mainly in blood vessels, but it can also occur between tunic cells (Tanaka and Watanabe, 1973; Tanaka, 1973). In *B. violaceus*, it appears that contact between allogeneic cells alone does not elicit rejection. In light of the phylogenetic position of this species, we suggest that the distribution of the allo-recognition site of botryllid ascidians has been extended from the blood cells to the sub-cuticular region, after which it has been lost except for the sub-cuticular region in *B. violaceus*.

In allogeneic fusion resulting from the contact of cut surfaces in *B. violaceus*, a natural separation of fused colonies was occasionally observed. This separation may be a manifestation of long term allo-recognition. If so, the separation may be comparable to that in *Botryllus scalaris* (Saito and Watanabe, 1982).

In xeno-grafts made at the cut surfaces between *B. vio-*

laceus and *B. simodensis*, necrosis occurred in both species. Thus, short term xeno-recognition is present between the two species.

A "single locus and multiple alleles model" has been proposed for the colony specificity of *B. primigenus* and *B. schlosseri*. (Oka and Watanabe, 1957, 1960, 1967; Sabbadin, 1962, 1982; Scofield *et al.*, 1982). According to this model, each colony has two alleles at one locus governing colony specificity, and colonies having at least one allele in common are fusible with one another. Mukai and Watanabe (1975) suggested that colony specificity in *B. simodensis* could also be explained by this model. The genetic system governing the colony specificity in *B. violaceus* is expected to be similar to this model, but further studies are required before reaching a definite conclusion.

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